SPECIFICATION AMENDMENTS

Description of the Drawings:

Figure 1 Predicted amino acid sequences of human class II histone deacetylases. The conserved residues of the catalytic domains are highlighted. (A) HDAC4 (SEQ ID NO: 2) (B) HDAC5 (SEQ ID NO: 4) (C) HDAC6 predicted amino acid sequences (SEQ ID NO: 6). Note that there are two putative catalytic domains (consisting of residues 215-287 and residues 610-683) in HDAC6. (D) Alignment of catalytic domains of ~~yeast HDA1p~~ (listed top to bottom) human HDAC1 (SEQ ID NO: 15), human HDAC4 (SEQ ID NO: 8), and human HDAC5 (SEQ ID NO: 9), ~~with both~~ catalytic regions ~~of~~ consisting of residues 215-287 of HDAC6 (SEQ ID NO: 10), catalytic region consisting of residues 610-683 of HDAC6 (SEQ ID NO: 74), and yeast HDA1p (SEQ ID NO: 16), The residues that are conserved in these HDACs as well as in acuC (*B. subtilis*, Accession 348052) and ASD (*M. ramosa*, Accession 3023317) are in bold type, and those residues that are conserved within the Class II human HDAC enzymes are boxed.

Figure 2. Expression analysis of novel human Class II HDAC family members. Multiple human tissue northern blots were probed to determine mRNA expression of HDAC4, HDAC5, and HDAC6. Blots were stripped and reprobed with β-actin cDNA to normalize for total mRNA. The position of molecular size markers is indicated to the left.

Figure 3. Class II HDAC enzymes deacetylate all four core histones *in vitro*. Recombinant FLAG-tagged HDACs were immunoprecipitated from transfected Jurkat cell extracts using α-FLAG antibody (Sigma). Immunopurified enzymes were incubated with radiolabeled core histones as described in Methods. (A) The HDAC activity was measured by scintillation counting of the released [^{3}H]-acetic acid. Where indicated, immunoprecipitates were preincubated with trichostatin A (Wako) prior to addition of histones. Each assay was performed in duplicate and averaged. (B) Substrate specificity of class II HDACs. Deacetylase reactions were separated by 20% SDS/PAGE and stained with Coomassie (top). The gel was treated with EnHance (National Diagnostics), dried and exposed to film (bottom). The identities of the core histones are indicated to the left. RbAp48 was transfected as a negative control.

Figure 4. The catalytic domains of HDAC6 function independently. The histidine residues homologous to H141 of HDAC1 in each of the catalytic domains (H216 and H611) were mutated to alanine by PCR overlap extension. The single and double mutants were FLAG-tagged and expressed in Tag-Jurkat cells. The enzymes were immunoprecipitated using α-FLAG antibodies (Sigma) and expression levels were compared by Western blotting (A). The mutant enzymes were then assayed for histone deacetylase activity as before (B).

Figure 5. Class II HDAC enzymes and HDAC1 are in different complexes *in vivo*. Recombinant FLAG-tagged HDACs were precipitated from transfected Jurkat cell extracts using α-FLAG antibody (Sigma), separated by SDS/PAGE and subjected to Western blot analysis. Blots were probed with (A) α-FLAG antibody (Sigma) to determine expression levels and (B) α-CHD4, α-mSin3A, α-MTA, α-HDAC1, α-HDAC3 and α-Rbp48 antibodies to determine if these proteins co-immunoprecipitated with the Class II HDAC enzymes.

Figure 6. Sequence analysis suggests that HDAC enzymes have diverged into two classes. (A) Alignment of human HDAC enzymes 1 through 6 with yeast Rpd3p, Hos1p, Hos2p, Hos3p, *M. ramosa* ASD, and *B. subtilis* acuC reveals the presence of seven conserved regions ("homology regions 1-7"), whose consensus sequences (class I consensus sequences for regions 1-7 are SEQ ID NOs 75, 17, 19, 20, 22, 23, and 24, respectively; class II consensus sequences for regions 2-5 and 7, are SEQ ID NOs 18, 76, 21, 77, and 78, respectively) differ between the two classes. Amino acids are represented by single letter codes; X represents any amino acid while Φ indicates a hydrophobic residue. NF = not found. Note that for homology region 7, Hos1p has consensus sequence SEQ ID NO: 25 rather than SEQ ID NO: 23. (B) A phylogenetic analysis suggests that the HDAC enzymes diverged from a common prokaryotic ancestor to form two classes of HDAC proteins. Proteins from three different phyla were examined. Prokaryotic proteins are preceded by (pro), yeast proteins are preceded by y, while human proteins are capitalized. Note that yHos3p does not correlate well with either HDAC class.

~~FIGURES 7A AND 7B. ASSOCIATION OF HDAC4 AND HDAC5 WITH TWO ISOFORMS OF 14-3-3~~ **Figures 7A and 7B. Association of HDAC4 and HDAC5 with two isoforms of 14-3-3**

A) Recombinant, FLAG-tagged HDAC1 and HDAC4 were transiently expressed in TAg Jurkat cells and immunoprecipitated using α-FLAG agarose (Sigma). Mock-transfected cells were used as a negative control. The immunopurified complexes were separated by SDS/PAGE and the proteins were visualized by silver stain. Two novel bands at 30 and 32 kDa in the HDAC4 immunoprecipitate were identified as 14-3-3 β and ε by peptide microsequencing analysis,
B) The association between HDAC4 and HDAC5 with 14-3-3 was confirmed by Western blot analysis. The recombinant FLAG-tagged proteins were immunoprecipitated with α-FLAG agarose and the purified complexes were separated by SDS/PAGE. The presence of HDAC3, 14-3-3 β and ε was confirmed by probing with specific antibodies (Santa Cruz).

~~FIGURES 8A AND 8B. NUCLEAR-CYTOPLASMIC SHUTTLING OF HDAC4 AND HDAC5 IS CORRELATED TO 14-3-3 EXPRESSION LEVELS~~ **Figures 8A and 8B. Nuclear-cytoplasmic shuttling of HDAC4 and HDAC5 is correlated to 14-3-3 expression levels**

~~A) RECOMBINANT HDAC4-EGFP AND HDAC5-EGFP WERE TRANSIENTLY EXPRESSED IN U2OS CELLS AND THE LOCALIZATION OF THE PROTEIN WAS OBSERVED BY FLUORESCENCE MICROSCOPY.~~
(A) Recombinant HDAC4-EGFP and HDAC5-EGFP were transiently expressed in U2OS cells and the localization of the protein was observed by fluorescence microscopy.

B) Overexpression of 14-3-3 β causes an increased cytoplasmic localization of HDAC4-EGPF. U2OS cells were transiently transfected with HDAC4-EGFP and either a control plasmid (pcDNA3.1, Invitrogen) or myc-tagged 14-3-3 β. Forty-eight hours post-transfection, the cells were fixed with paraformaldehyde and probed with an α-myc antibody (Upstate Biotechnology) and an α-mouse Ig Texas Red conjugated secondary antibody (Sigma). The DNA was stained with Hoechst dye (Molecular Probes).

Figures 9A and 9B. Phosphorylation-dependent binding of 14-3-3 to HDAC4 and HDAC5
A) Association of HDAC4 and HDAC5 with 14-3-3 and HDAC3 is dependent on the phosphorylation state of the proteins. HDAC4-FLAG and HDAC5-FLAG were transiently

expressed in TAg Jurkat cells. Forty-eight hours post-transfection, the cells were treated for 1.5 hours with staurosporine and calyculin A. The immunopurified HDAC4-FLAG and HDAC5-FLAG complexes were subjected to Western blot analysis and tested for HDAC activity, as described in the experimental procedures. The HDAC activity was measured by scintillation counting of the released [^{3}H]-acetic acid.

B) The binding of 14-3-3 to HDAC4 prevents interaction with importin α. Forty-eight hours after transfection with HDAC4-FLAG, TAg Jurkat cells were treated with staurosporin or calyculin A for 1.5 hours. HDAC4-FLAG was immunopurified and subjected to Western blot analysis with α-importin α antibodies (Transducin Laboratories).

Figure 10. Schematic representation of HDLP-TSA complex interactions.

The TSA HDAC inhibitor is shown bound to HPLP (C7-C15 of TSA are labeled). The surrounding HDLP residues are ~~labelled~~ labeled and the corresponding HDAC1 active site residues are indicated below in parentheses. The Zn^{2+} cation in the active site of HDLP is showns as a filled circle. Thatched semi-circles indicate van der Waals contacts between hydrophobic protein resides and TSA, while hydrogen bonds are shown as dashed lines.

Figure 11. Proposed catalytic mechanism for deacetylation of acetylated lysine.

HDLP active site residues and their proposed HDAC counterparts (in ~~paranthesespp~~parentheses) are ~~labelled~~ labeled.

Figure 12. General structural feature of HDAC inhibitors.

The general structural features of HDAC inhibitors include a Cap group which can be varied to optimize inhibition of a particular HDAC or class of HDACs, an aliphatic chain and a functional group which interacts with the active site of the HDAC.

Figure 13. Alterations in residues contacting TSA in human HDACs.

The residues contacting TSA in HDLP are those which are both shaded and ~~labelled~~ labeled with a consensus residue (i.e. P22 and Y91 residues in the rim of channel, G140, F141, F198, and L265 residues in the channel, and H131, 132, D166, D168, H170, D258, and Y297 residues in the active site) is shown. The surrounding resides that differ between class I and class II HDACs

are shaded and ~~labelled~~ labeled with an asterisk (*). The residues on the rim of the channel for HDLP, HDAC1, HDAC2, HDAC3, HDAC8, HDAC5, and HDAC7 are listed as SEQ ID NOs 26-33, respectively. The residues in the channel for HDLP, HDAC1, HDAC2, HDAC3, HDAC8, HDAC5, HDAC6(a) (residues 215-287), HDAC6(b) (residues 610-683), and HDAC7 are listed as SEQ ID NOs 34-43, respectively. Residues corresponding to residues 126-133 of the HDLP active site for HDLP, HDAC1, HDAC2, HDAC3, HDAC8, HDAC5, HDAC6(a) (residues 215-287), HDAC6(b) (residues 610-683), and HDAC7 are listed as SEQ ID NOs 44-53, respectively. Residues corresponding to residues 164-173 of the HDLP active site for HDLP, HDAC1, HDAC2, HDAC3, HDAC8, HDAC5, HDAC6(a) (residues 215-287), HDAC6(b) (residues 610-683), and HDAC7 are listed as SEQ ID NOs 54-63, respectively. Finally, residues corresponding to residues 257-259 and 294-297 of the HDLP active site for HDLP, HDAC1, HDAC2, HDAC3, HDAC8, HDAC5, HDAC6(a) (residues 215-287), HDAC6(b) (residues 610-683), and HDAC7 are listed as SEQ ID NOs 64-73, respectively.

Figure 14. HDAC inhibitor small molecule libraries.
The generalized structures of two representative HDAC inhibitor compound libraries are shown.

Figure 15. Representative positive from HDAC6 screen of printed library.
Cy5HDAC was used to screen~~t he~~ the printed 1,3-dioxane library. A representative positive (compound 4-P9) from this screen is shown.

Figure 16. ~~Affect~~ Effect of representative compounds on HDAC1 and HDAC6 activity.

Figure 17. Retesting of resynthesized 11-A15.